

Theophylline Synergizes With Chlorambucil in Inducing Apoptosis of B-Chronic Lymphocytic Leukemia Cells

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We tested the effects of theophylline, a phosphodiesterase inhibitor inducing intracellular accumulation of cyclic adenosine monophosphate (cAMP), on malignant B cells from 15 patients with B-chronic lymphocytic leukemia (B-CLL). We observed a large increase in apoptotic cell numbers (mean, 90% \pm 20% in medium alone) in the presence of theophylline (100 μ g/mL) or chlorambucil (10 μ mol/L) after 72 hours of incubation. Maximal apoptosis (90%) was reached after 36 hours when the two drugs were used together at fourfold lower concentrations, indicating a synergistic effect; no effect was observed with normal B cells, suggesting that the combination might have therapeutic interest. Chlorambucil induced intracellular Ca^{++} influx, pointing to the involvement of two signaling pathways that might explain its syn-

ergy with theophylline through their effects on oncogenes. The expression of bcl-2 protein, a proto-oncogene inhibiting apoptosis, decreased after incubation with the drugs, while c-myc, recently described as having a potent role in apoptosis, was overexpressed. For p53 we observed an overexpression in the presence of chlorambucil or both theophylline-chlorambucil and a decrease after theophylline incubation. Chlorambucil- and theophylline-induced apoptosis was partially inhibited by interleukin-4 (IL-4), which also abrogated the effects on oncogene expression. These results provide insight into the mechanisms underlying B-CLL apoptosis and suggest that the theophylline-chlorambucil combination may be of therapeutic value in this setting.
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APOPTOSIS OR PROGRAMMED cell death plays a key role in the selection and differentiation of lymphoid cells.^{1,2} Defective apoptosis may be involved in the onset of several malignancies, including those of the hematopoietic B-cell system.^{3,4} Induction and acceleration of apoptosis have thus become major aims in new pharmacological approaches to cancer. In this study, we analyzed apoptosis of chronic lymphocytic leukemia of B-cell origin (B-CLL) *in vitro*.

Apoptosis of B-CLL may occur spontaneously⁵ and in response to stimuli such as calcium ionophore, specific antibodies binding to membrane associated IgM, steroids,⁶ purine nucleoside analogs (fludarabine and 2-chlorodeoxyadenosine),⁷ and chlorambucil.⁸ Phorbol esters, which induce protein kinase C phosphorylation, prevent apoptosis in the presence of the above compounds, with the exception of chlorambucil.^{6,7,9} We recently reported that a methylxanthine derivative, theophylline, can also elicit B-CLL cell apoptosis through a cyclic adenosine monophosphate (cAMP) pathway.¹⁰ Cytokines can induce (interleukin [IL]-10¹¹ and IL-5¹²) or prevent (IL-4¹³ and interferons γ ¹⁴ and α ¹⁵) apoptosis of B-CLL cells, suggesting that these molecules modulate this process *in vivo*.

The precise mechanisms underlying apoptosis remain unknown. In certain conditions, dysregulation of the p53, c-

myc, and bcl-2 oncogenes can cause apoptosis in human lymphoid malignancies.¹⁶ Bcl-2 gene overexpression has been found to protect cells against several apoptotic stimuli.^{4,17,18} The high level of bcl-2 expression generally observed in CLL (without gene rearrangement in most cases) probably plays a critical role in the physiopathology of this disease.^{19,20} There is evidence that the p53 tumor-suppressor oncogene is involved in apoptosis,²¹ and mutation of this gene has been described in about 15% of patients with poor-prognosis B-CLL.²² Abnormal c-myc oncogene expression enhances cell susceptibility to various apoptosis-inducing agents, probably by interacting with p53.^{20,23,24} It has also been demonstrated that c-myc expression can result in mitosis or apoptosis, depending on the availability of other critical stimuli.²⁵

We studied the *in vitro* apoptosis of peripheral blood cells from 15 patients with B-CLL in the presence of chlorambucil (the agent most commonly used to treat this disease) and/or theophylline. Our data point to synergy between these two drugs through different signaling pathways and on modulation of oncogenes such as p53, c-myc and bcl-2.

MATERIALS AND METHODS

Isolation of leukemic and normal B cells. Peripheral blood from 15 patients with B-CLL and from three healthy donors was obtained with their informed consent. B-CLL was diagnosed on the basis of morphologic and immunologic criteria. Mononuclear cells were isolated by density gradient centrifugation ($d = 1.077$ g/L, Ficoll-Paque, Seromed, Berlin, Germany). Human B cells were isolated by positive selection with anti-CD19-coated magnetic beads (Dynabeads M-450 Pan-B, Dynal, Oslo, Norway) as previously described.²⁶ Briefly, cells were incubated for 30 minutes at 4°C with Dynabeads, with a target to bead ratio of 10:1. Rosetted cells were captured with a magnet, and nonrosetted cells were removed by suction. The cell rosettes were washed five times and resuspended in RPMI containing 10% fetal calf serum (FCS). Beads were removed from positively selected cells by incubating with Fab-antiserum (Dynal) for 45 minutes at 4°C and placing the suspension on a magnet. We obtained highly purified B cells (>98%) with less than 0.1% of T cells, 0.1% of natural killer cells and 0.5% of monocytes, as shown by means of flow cytometry (immunofluorescence staining with anti-CD2, anti-CD3, anti-CD56, and anti-CD14 antibodies, all from Immunotech, Marseille-Luminy, France).

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Cell culture conditions. Freshly isolated B cells (5×10^6 mL) were cultured in RPMI 1640 medium (Seromed) supplemented with penicillin, streptomycin, glutamine, β_2 mercapto-ethanol, and 10% FCS. Cultures were supplemented with various factors, as follows: IL-2 (100 U/mL) was from Eurocetus (Amsterdam, The Netherlands), IL-4 (10 ng/mL) was a generous gift from J. Banchereau (Schering-Plough, Dardilly, France), N^6 2'-O-Dibutyryl adenosine 3',5'-cyclic monophosphate, ionomycin, and Bapta-AM were from Sigma (St Louis, MO), Rp-cAMP was from Boehringer Mannheim (Meylan, France), theophylline was from PCH (Paris, France), and chlorambucil was a generous gift from A. Sirito (Techni-Pharma, Monaco, France). For B-cell proliferation, Western blotting, and DNA gel fragmentation experiments, cells were incubated with various compounds for 36 and 72 hours or for 6 and 24 hours for cyclic-AMP measurement and Northern blot analysis, respectively.

Calcium measurement. Intracytoplasmic free calcium was measured with Fura-2 acetoxymethyl ester (Fura-2/AM). B cells (6×10^6) were washed once and loaded with $3 \mu\text{mol/L}$ of Fura-2/AM in $300 \mu\text{L}$ of 50 mmol/L Hepes buffer at pH 7.4 supplemented with 125 mmol/L NaCl, 1 mmol/L CaCl_2 , 0.5 mmol/L MgCl_2 , 5 mmol/L KCl, 1 mmol/L Na_2HPO_4 , and 1 mg/mL of glucose (all from Sigma). After 20 minutes at 37°C , the cell suspension was diluted with 6.7 mL of the same buffer and then reincubated for 20 minutes at 37°C . Cells were then rapidly centrifuged and pellets were resuspended in 2 mL of buffer for reading. The fluorescence of the cell suspension was monitored with a Perkin-Elmer LS5B luminescence spectrometer (Perkin-Elmer, Bois-d'Arcy, France). Graphic representations of Ca^{++} were computed using the equation: $\text{Ca}^{++} = 225 \times (\text{R-Rmin})/(\text{Rmax-R}) \times \text{Sf380/Sb380}$.²⁷

Cyclic-AMP measurement. B-CLL cells (5×10^6 mL) were resuspended in RPMI 1640 medium supplemented with 200 $\mu\text{mol/L}$ of the phosphodiesterase inhibitor IBMX (Sigma) and incubated at 37°C for 15 minutes. Next, 1×10^6 cells were distributed into Eppendorf microtubes, supplemented with 50 μL of stimuli and incubated for 15 minutes to 6 hours at 37°C in a water bath. The reaction was stopped by adding 25 μL of 35% perchloric acid (Sigma) and immediate cooling on ice. The tubes were allowed to stand at 0°C for 15 minutes and were then spun at 3,000g for 15 minutes. The pellets were discarded and the supernatants were neutralized with potassium hydroxide (KOH) and kept at -80°C until cAMP determination by means of a specific radioimmunoassay (Amersham) as recommended by the manufacturer. Data are expressed as pmol per 10^6 cells.

Protein extraction and Western blot analysis. Bcl-2, wild-type p53, and c-myc protein expression were analyzed by Western blot and compared case by case relative to expression before treatment. Whole-cell lysates were prepared by pelleting 0.5 mL of cell suspension (1×10^7 cells) for 30 seconds in a microcentrifuge, aspirating the supernatants, and adding 50 μL of lysis buffer containing 20 mmol/L Tris, 1 mmol/L EDTA, 140 mmol/L NaCl, 1% Nonidet P40 (NP40), 1 mmol/L orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 50 U/mL aprotinin for 30 minutes with occasional agitation. They were then centrifuged for 30 minutes at 14,000 rpm at 4°C , and the supernatants were recovered. Protein was quantified in each supernatant by a colorimetric method according to Bradford,²⁶ and the samples were diluted in 50 μL of sample buffer (10 mL glycerol, 0.5 mol/L Tris/HCl, 10% sodium dodecyl sulfate (SDS), 10 mg bromophenol blue, 5% β_2 mercapto-ethanol qsp, 50 mL H_2O , pH 6.8; all from Sigma).

Each sample was boiled for 3 minutes, and 30 or 60 μg of protein was loaded onto 10% polyacrylamide gels and electrophoresed for 3 hours at 25 mA. Protein was then transferred to a nitrocellulose membrane (Immobilon, Millipore, Bedford, MA) for 75 minutes at 400 mA in buffer containing 92 mmol/L glycine, 25 mmol/L Tris, 0.5% SDS, pH 8.3. Blots and polyacrylamide gels were routinely

stained with Coomassie blue to check the transfer efficiency. The nitrocellulose membrane was consecutively incubated with anti-bcl-2 antibody (Dako), anti-p53 antibody (UBI, Lake Placid, NY) and anti-c-myc antibody (Tebu) in 1% Tween-Tris buffer saline (T-TBS) gelatine (Prolabo, Paris, France) for 1 hour at room temperature and was then washed three times for 10 minutes in 0.1% T-TBS (rinsing buffer). The blots were also stained with anti-PLC γ 1 antibody (UBI) to check protein deposition. Blots were incubated with peroxidase-conjugated goat antimouse IgG2b or goat antirabbit IgG (Biorad, Richmond, CA) secondary antibodies for 40 minutes at room temperature, washed three times in rinsing buffer, dried, and revealed with chromogen substrate (ECL, Amersham, Buckinghamshire, UK) for 2 minutes. Autoradiography was performed with Cronex X-ray film (Dupont de Nemours, Les Ulis, France). Prestained molecular weight markers (Biorad) were included in each gel. Density was quantified by means of laser densitometry (Molecular dynamics, Sunny Vale, CA).

mRNA analysis. Northern blot analysis was performed as previously described.²⁸ Briefly, total RNA was extracted from cells by treatment with RNAlplus (Bioprobe Systems, Montreuil-sous-Bois, France) and 10 μg (bcl-2) or 20 μg (c-myc and p53) was electrophoresed through an 1% agarose gel and transferred to nylon membranes. Blots were then hybridized to human DNA probes radiolabeled to a specific activity of 1×10^6 cpm/ng with dCTP α -³²P (bcl-2 and c-myc) or ATP γ -³²P (p53), using a commercial labeling kit. Levels of bcl-2 were determined with the *EcoRI/HindIII/BamHI* insert from plasmid pB16, c-myc with the *Sac I* insert from plasmid pKH47 and p53 with specific DNA oligonucleotide (Euromedex, Souffelweyersheim, France).

Cell apoptosis. DNA fragmentation was analyzed as follows: at the times indicated 0.5-mL aliquots of cell suspension were lysed by adding 1 mL of ice-cold buffer containing 0.5% triton X-100, 20 mmol/L Tris, 1 mmol/L EDTA pH 7.4 before centrifugation for 20 minutes at 13,000 rpm in a microcentrifuge. DNA in the supernatant (DNA fragments) was precipitated in isopropanol (vol:vol) buffer containing 5 mol/L NaCl at -20°C for 6 hours and resuspended in buffer containing 10 mmol/L Tris, 1 mmol/L EDTA pH 8.0 before electrophoresis for 3 hours at 60 V in 1.8% agarose gels. The DNA fragments were then visualized under ultraviolet (UV) light (312 nm) after staining the gels with ethidium bromide. At the same time of incubation for indicated culture conditions, apoptotic cells were detected by using an in situ apoptosis detection kit (Boehringer Mannheim, Meylan, France) as previously described.¹⁰ For each of the 15 patients, 1,000 cells were counted and the percentage of positive cells was recorded.

RESULTS

Theophylline and chlorambucil consistently induce B-CLL cell apoptosis, are synergistic, and their effect is reversed by IL-4. We have recently demonstrated that theophylline induces apoptosis of B-CLL cells in vitro.¹⁰ In the present study, we analyzed the concentration dependency of this effect and compared it with the apoptotic effect of chlorambucil. To establish whether apoptosis was effectively the observed mode of cell death, cells from the 15 patients were analyzed for DNA fragmentation, a marker of apoptosis. DNA strand breaks were detected by in situ dUTP labeling, agarose gel electrophoresis (Fig 1) and propidium iodide staining in flow cytometry (data not shown), and a strong correlation was obtained between the results in these various methods. As shown in Table 1, the mean proportion of B-CLL cells showing apoptosis after 72 hours of culture with theophylline or chlorambucil was respectively 87% (range,

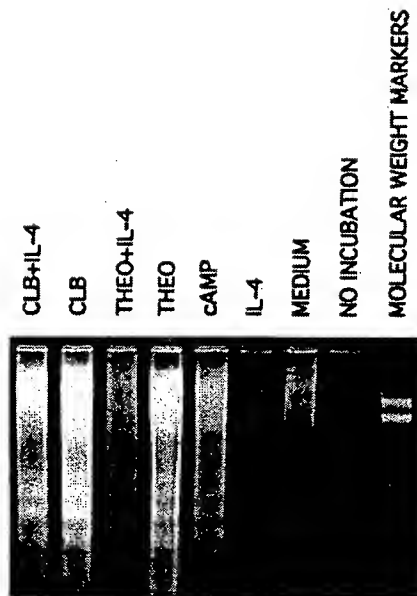


Fig 1. Agarose gel (1.8%) electrophoresis of DNA extracted from B-CLL cells before and after 72 hours of incubation in various culture conditions (medium, 10 ng/mL IL-4, 100 mmol/L cAMP, 100 μ g/mL theophylline (THEO) and 10 μ mol/L chlorambucil (CLB) alone or in combination with IL-4 as indicated in the figure. Representative data from patient no. 12 are shown.

Table 1. Percentage of Apoptotic B-CLL Cells in the Presence of Various Factors

B-CLL No.	Culture Conditions					
	Medium	THEO	CLB	IL-4	THEO + IL-4	CLB + IL-4
1	14	91	89	4	51	64
2	20	93	92	8	47	72
3	9	85	91	3	40	58
4	17	96	95	3	62	67
5	14	77	ND	5	16	ND
6	13	94	88	2	45	74
7	3	75	ND	1	30	ND
8	14	94	95	8	52	60
9	17	87	94	7	20	58
10	31	86	ND	15	30	ND
11	13	90	92	6	42	65
12	16	93	91	4	45	57
13	17	77	ND	3	33	ND
14	25	81	92	10	51	63
15	8	83	87	3	16	41
Control*	5	9	9	2	3	4

B-CLL cells were cultured during 72 hours in presence of various factors (THEO, 100 μ g/mL, CLB, 10 μ mol/L, IL-4, 10 ng/mL, THEO + IL-4, 100 μ g/mL added to IL-4 10 ng/mL, CLB + IL-4, 10 μ mol/L added to IL-4 10 ng/mL or in medium alone). Percentages of apoptotic cells were recorded as described in Materials and Methods.

Abbreviations: THEO, theophylline; CLB, chlorambucil; IL-4, interleukin-4; ND, not done.

* Mean percentage from 5 normal B-cell samples.

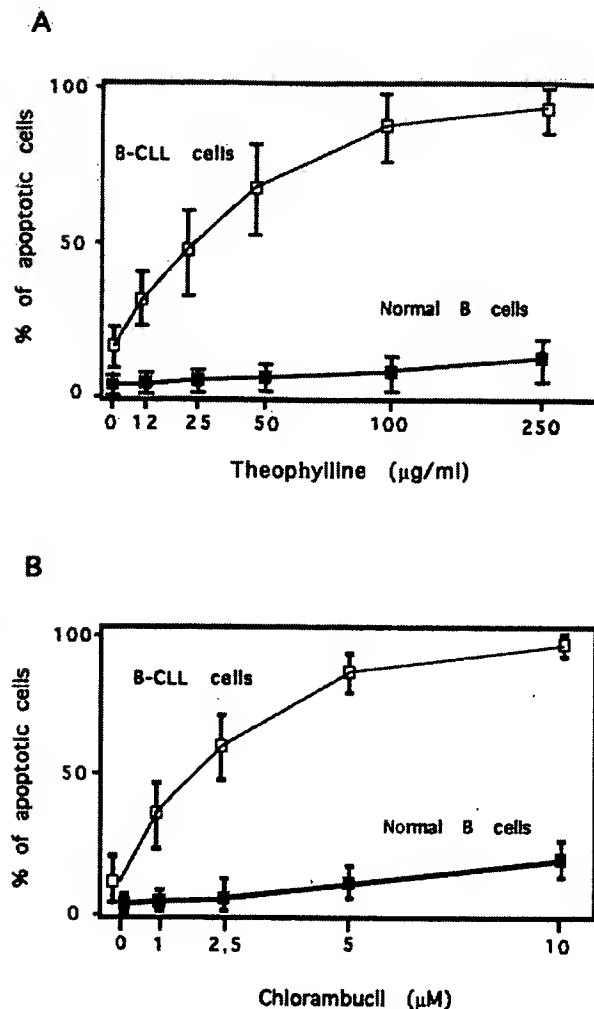


Fig 2. (A) Dose-dependent apoptosis of B-CLL cells with theophylline (from 12 to 250 μ g/mL). Data are presented as means \pm SEM of at least three experiments each for all 15 B-CLL cases and five normal donors. (B) Dose-dependent apoptosis of B-CLL cells with chlorambucil (from 1 to 10 μ mol/L). Data are presented as means \pm SEM of at least three experiments each for all 11 B-CLL cases and four normal donors.

77% to 96%) and 92% (range, 87% to 95%) compared with 16% (range, 3% to 25%) in medium alone. No significant percentage of apoptotic cells was observed in normal B cells used as control. This effect was concentration-dependent, and the highest apoptosis values were obtained with 100 μ g/mL theophylline and 10 μ mol/L chlorambucil (Fig 2A and B). Cells were then incubated with optimal and suboptimal concentrations of chlorambucil and theophylline and apoptosis was quantified. We observed a constant synergy between the two drugs at any concentration and any time (Fig 3A and B). Indeed, the percentage of apoptotic cells reached 50% when the two drugs were used in combination at very low concentrations, while the same results were obtained with theophylline 20-fold higher and chlorambucil 10-fold higher concentrations. Furthermore, maximum

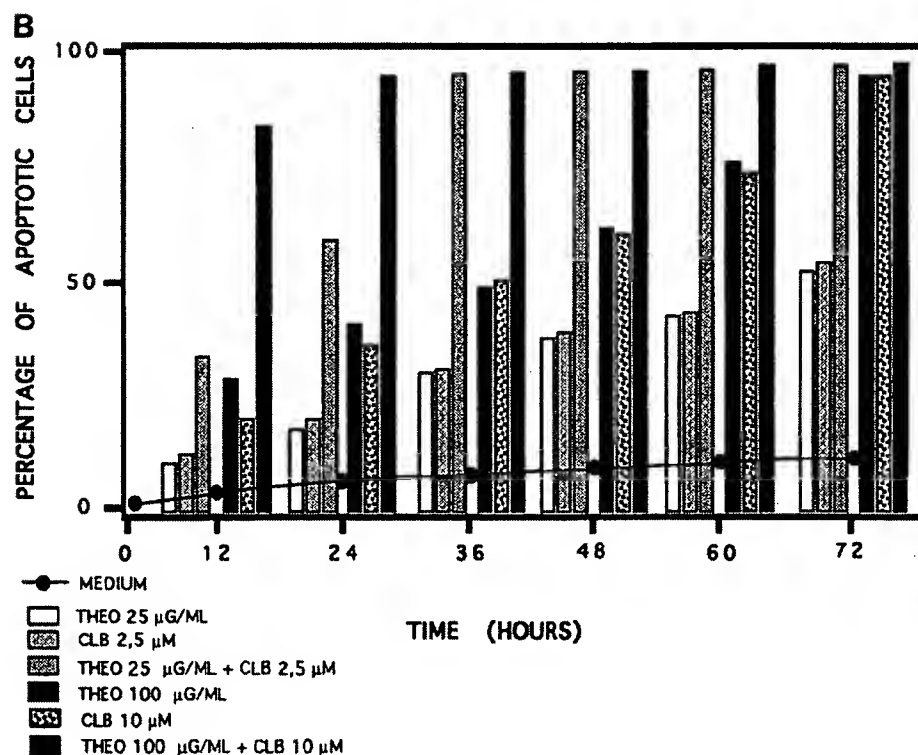
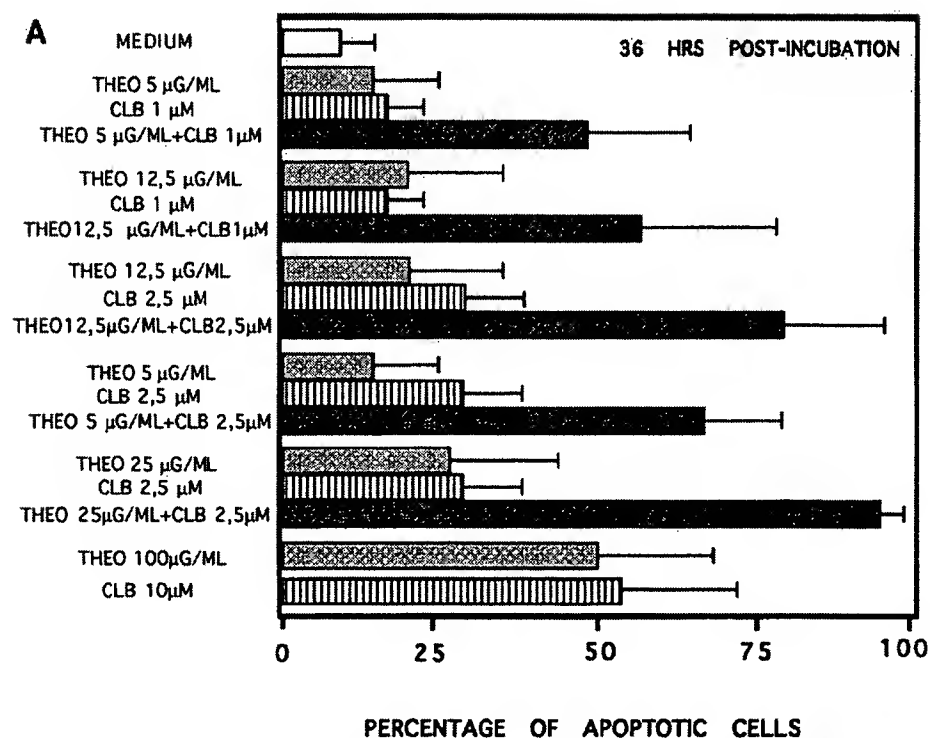


Fig 3. (A) Synergistic effect of theophylline on chlorambucil-induced B-CLL apoptosis. Percentages of apoptotic cells are the mean from 11 B-CLL cases and were obtained at 36 hours post-incubation with increasing doses of theophylline (THEO) and chlorambucil (CLB) as indicated in the figure. (B) Synergistic effect of theophylline on chlorambucil-induced B-CLL apoptosis. Percentages of apoptotic cells are the mean from 11 B-CLL cases and were observed every 12 hours from 0 to 72 hours post-incubation with different doses of theophylline (THEO) and chlorambucil (CLB) as indicated in the figure.

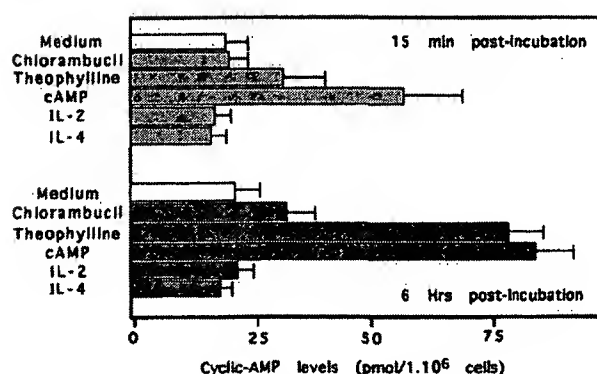


Fig 4. Induction of intracellular cAMP levels by 100 μ g/mL theophylline after 15 minutes and 6 hours of incubation compared with various culture conditions (medium, 10 μ mol/L chlorambucil, 100 mmol/L cAMP, 100 U/mL IL-2 and 10 ng/mL IL-4). Data are presented as means \pm SEM of at least three experiments in six B-CLL cases.

apoptosis (90%) was reached at 36 hours instead of 72 hours when each drug was used alone. The same drug combination had little, if any, effect on apoptosis of normal B cells in similar culture conditions (mean, 10%; range, 6% to 15%).

The addition of IL-4 (10 ng/mL) to theophylline-treated cultures significantly reduced apoptosis in 12 of 15 B-CLL samples (mean, -59%; range, -43% to -89%) and totally inhibited apoptosis in the remaining three samples (>-95%, patients no. 5, 9, and 10). In contrast, chlorambucil-induced apoptosis was only partially reduced (mean, -36%; range, -18% to -58%) even when an IL-4 concentration of 20 ng/mL was used (Fig 1 and Table 1).

Theophylline-induced apoptosis is associated with intracellular cAMP accumulation and chlorambucil-induced apoptosis with calcium influx. Theophylline is a well known inhibitor of intracellular cyclic nucleotide phosphodiesterase (CN-PDE) resulting in accumulation of cAMP.²⁹ The mechanism by which chlorambucil, an alkylating agent, elicits apoptosis has not been yet elucidated. We thus investigated whether intracellular cAMP accumulation was induced by theophylline and/or chlorambucil in B-CLL cells, together with the potential role of this second messenger in apoptosis. In the six B-CLL cases tested, theophylline (100 μ g/mL) induced a large increase in intracellular cAMP levels after 6 hours, ranging from 75 to 85 pmol/10⁶ cells; identical results were obtained with dibutyryl cAMP, a cAMP agonist, used as positive control; cAMP levels in the presence of medium alone were 16 to 20 pmol/10⁶ cells. In contrast, chlorambucil (10 mmol/L) induced only moderate cAMP accumulation (range, 27 to 30 pmol/10⁶ cells). All these results are shown in Fig 4.

Calcium mobilization has been described as an early event in the apoptosis induced by glucocorticoids and fludarabine.^{7,16,30} Chlorambucil stimulation (10 mmol/L) rapidly (<1 minute) induced a calcium influx (>500 nmol/L) in B-CLL cells, whereas theophylline had no effect on intracellular calcium levels, even at concentrations as high as 1 mg/mL. Successive additions of chlorambucil 5 μ mol/L induced an increase of intracellular calcium level up to a plateau (500

nmol/L). Chelation of extracellular free calcium by EGTA (3 mmol/L) induced a decrease of intracellular calcium level reversed by addition of CaCl_2 (5 mmol/L; see Fig 5A) showing evidence for calcium channel integrity. A slight mobilization of intracellular calcium from endoplasmic reticulum stores was observed with chlorambucil after pretreatment with EGTA. CaCl_2 addition resulted in a high calcium level increase (Fig 5B). All these data demonstrated that chlorambucil induced in B-CLL cells both a calcium mobilization and a calcium influx.

To determine the role of these second messengers in B-CLL apoptosis, we treated cells with agonists and antagonists of cAMP and calcium pathways. Rp-cAMP, a cAMP antagonist, inhibited up to 30% of theophylline-induced apoptosis, while Bapta-AM, an intracellular calcium chelator, only partially reversed the effect of chlorambucil (mean, -15%) (data not shown). These data indicated that cAMP and calcium were only partially involved in the induction of apoptosis by theophylline and chlorambucil. These findings

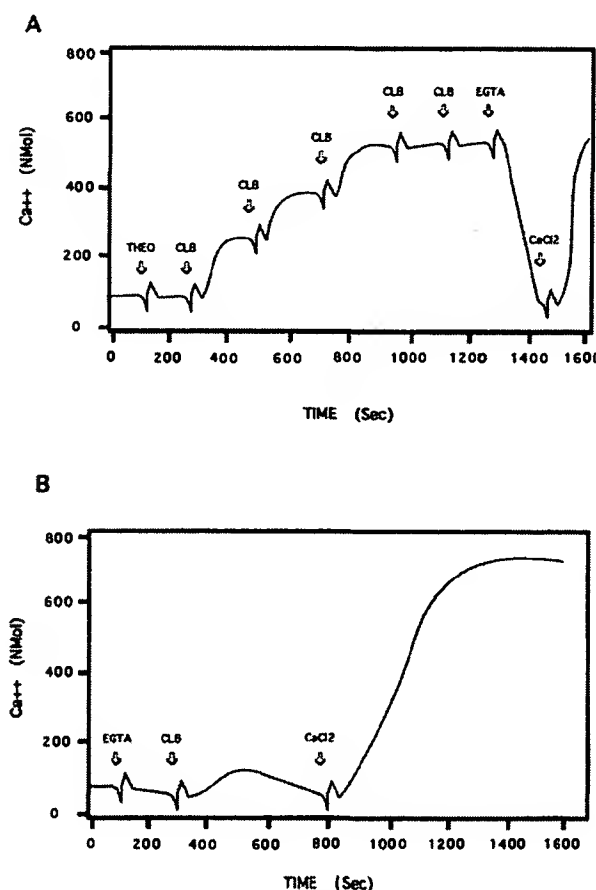


Fig 5. (A) Induction of Ca^{++} influx by successive additions of 5 μ mol/L chlorambucil (CLB). Theophylline (THEO) (100 μ g/mL) had no effect in the same experiment. EGTA (3 mmol/L) and CaCl_2 (5 mmol/L) have been used as chelator and ionophore, respectively. (B) Mobilization of intracellular Ca^{++} stores induced by 10 μ mol/L chlorambucil (CLB). EGTA (3 mmol/L) and CaCl_2 (5 mmol/L) have been used as chelator and ionophore, respectively.

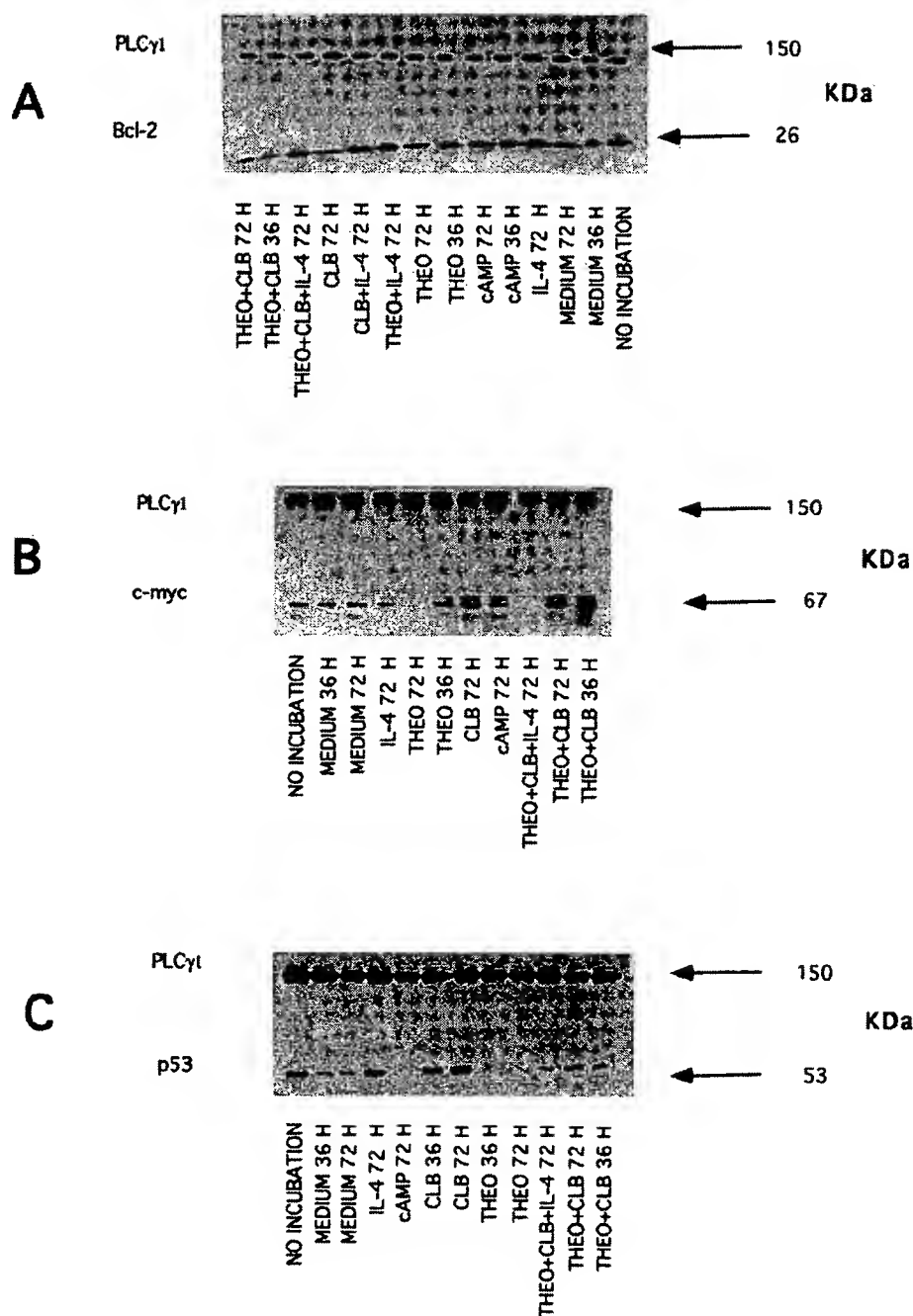


Fig 6. Western blot analysis. Proteins were extracted from B-CLL cells before and after incubation in various culture conditions at specified times: medium, 10 ng/mL IL-4, 100 mmol/L cAMP, 100 μ g/mL theophylline (THEO) and 10 μ mol/L chlorambucil (CLB) alone or in combination. The relative protein level was determined by Western blot analysis as described in Materials and Methods. Each lane was loaded with 30 μ g/mL (bcl-2) or 60 μ g/mL (c-myc and p53) of total protein. Blots were also hybridized with anti-PLC γ 1 as protein deposit control. Data from a representative case of 11 (patient no. 9 for bcl-2 and c-myc protein expression; patient no. 15 for p53 protein expression). (A) bcl-2 protein expression. (B) c-myc protein expression. (C) p53 protein expression.

were supported by experiments in which cell incubation with dibutyl cAMP (100 mmol/L), a cAMP analog, or ionomycin (1 μ mol/L), a calcium ionophore, resulted in lesser B-CLL cell apoptosis than observed with theophylline or chlorambucil, respectively (not shown). These data suggest the possible involvement of additional transduction pathways of B-CLL apoptosis induced by these drugs.

Theophylline and chlorambucil inhibit bcl-2 expression in B-CLL cells. Overexpression of bcl-2 oncogene is commonly observed in lymphoma and chronic leukemia of B-

cell origin³¹ and is known to downregulate apoptosis of these cells.^{30,32} We thus studied the influence of theophylline and chlorambucil on the degree of bcl-2 expression in B-CLL cells from the 15 patients. As shown in Fig 6A, treatment of leukemic cells with each drug resulted in significant and constant downregulation of intracellular bcl-2 protein expression. Compared with levels before treatment, the decrease was -32% (range, -10% to -55%; $P < .01$) after 72 hours in the presence of theophylline and -35% (range, -12% to -60%; $P < .01$) after the same time with chloram-

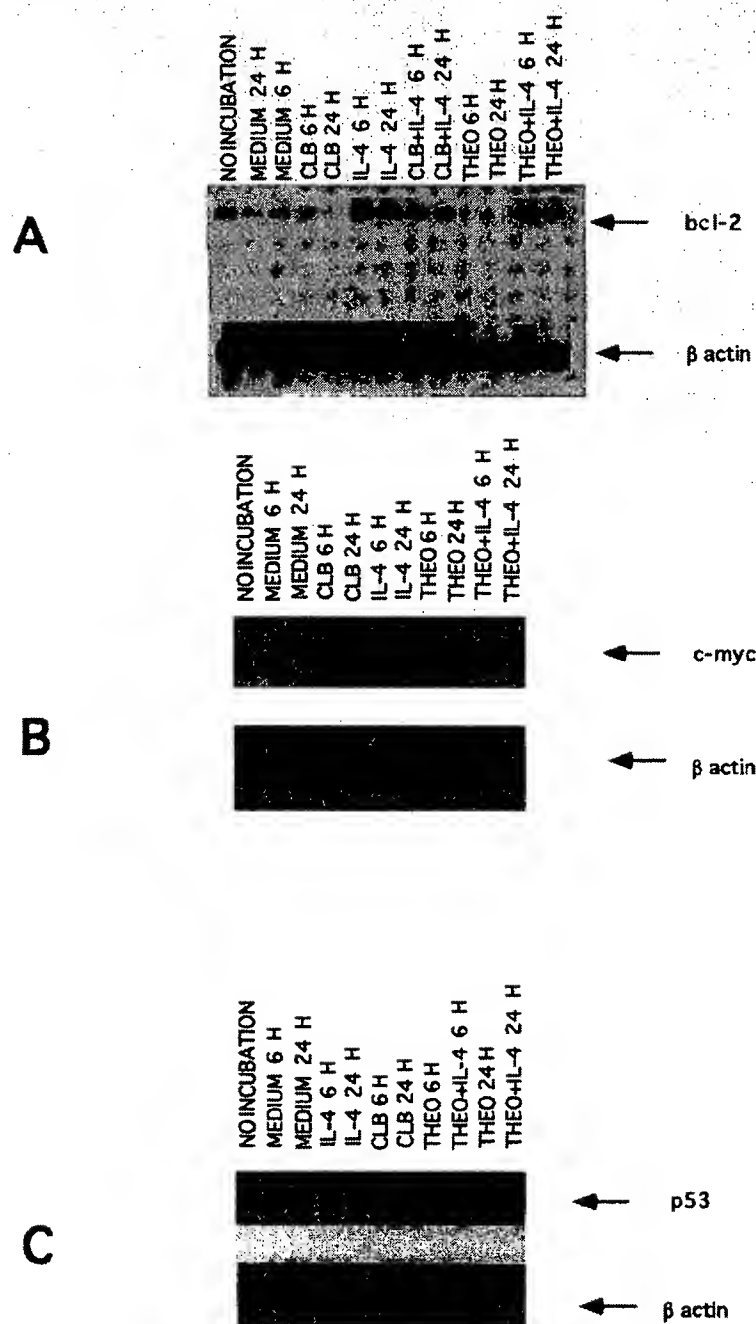


Fig 7. Northern blot analysis. RNA were extracted from B-CLL cells before and after incubation in various culture conditions at specified times: medium, 10 ng/mL IL-4, 100 mmol/L cAMP, 100 μ g/mL theophylline (THEO) and 10 μ mol/L chlorambucil (CLB) alone or in combination. Northern blot was performed on total RNA (10 μ g per lane for bcl-2, 20 μ g per lane for c-myc and p53 analysis). The blot was stripped and hybridized with reported gene probe. Data from a representative case of 8 (patient no. 9 for bcl-2 and c-myc protein expression; patient no. 15 for p53 protein expression). (A) bcl-2 mRNA expression. (B) c-myc mRNA expression. (C) p53 mRNA expression.

bucil. Similar results were observed after 36 hours of incubation with both drugs. A smaller decrease was observed after incubation with dibutyl-*c*-AMP (mean, -20%; range, -6% to -35%; $P < .03$). These data were confirmed by a fall in bcl-2 mRNA after as little as 6 hours of incubation either with theophylline or chlorambucil and its abrogation after 24 hours (Fig 7A). The antiapoptotic effect of IL-4 was corroborated by a clear upregulation of intracellular bcl-2 expression (Fig 6A).

Theophylline and chlorambucil induce c-myc overex-

pression in B-CLL cells. As the c-myc oncogene has been suggested to play a pivotal role in proliferation and apoptosis,^{23,24} we studied the expression of this proto-oncogene before and after cell treatment with theophylline and chlorambucil. Theophylline induced transient expression of c-myc mRNA after 6 hours of incubation, which ceased within 24 hours; the same c-myc expression pattern was observed with chlorambucil (Fig 7B). As shown in Fig 6B, theophylline led to a significant increase in c-myc protein levels at 36 hours (mean, +50%; range, +20% to +75%; P

< .01) with total abrogation at 72 hours (when cell apoptosis was complete). An increase was also obtained with chlorambucil alone or combined with theophylline at 72 hours (mean, +55% and +70%; range, +25% to +90% and +38% to +100%, respectively; $P < .01$) and, to a lesser extent, dibutyryl-cAMP (mean, +15%; range, +10% to +28%; $P < .01$). These findings were supported by mRNA studies and pointed to a role of c-myc induction in B-CLL apoptosis in the presence of theophylline and chlorambucil. IL-4 down-regulated c-myc protein expression in theophylline- and chlorambucil- pre-treated cells (Fig 6B).

Theophylline and chlorambucil differ in their effect on p53 expression. As the p53 oncogene appears to play a role in controlling hematopoietic cell apoptosis,²¹ we analyzed its expression during apoptosis induced by theophylline and/or chlorambucil in the 15 B-CLL samples. As shown in Fig 6C, theophylline dramatically reduced intracellular levels of p53 (mean, -68%; range, -40% to -95%; $P < .01$), an effect totally mimicked by dibutyryl-cAMP (100 mmol/L) (mean, -56%; range, -39% to -75%; $P < .03$). In contrast, chlorambucil- and in a lesser extent theophylline-chlorambucil combination-induced apoptosis was related to a significant increase in intracellular p53 levels (Fig 6C) (mean, +45% and +20%; range, +22% to +70% and +12% to +33%, respectively; $P < .01$). These data were confirmed by Northern blot analysis (Fig 7C). P53 protein expression was also reversed by IL-4; intracellular levels after treatment with this cytokine were similar to those observed before culture (Fig 6C).

It is of note that bcl-2, c-myc, and p53 protein levels studied in normal B cells from two healthy donors were very low before culture and not significantly modified after incubation with theophylline and/or chlorambucil (data not shown).

DISCUSSION

Many anticancer agents have been shown to induce apoptosis of malignant cells in vitro, but the relevance of these effects to in vivo activity was uncertain and the underlying biochemical events ill-defined. We show that suboptimal concentrations of both chlorambucil and theophylline strongly induce apoptosis of B-CLL cells, as previously described for 2-chlorodeoxyadenosine, fludarabine, steroids, and chlorambucil alone; all these drugs have proven clinical efficacy. The poor effectiveness of the same suboptimal concentrations of chlorambucil and theophylline in inducing apoptosis of normal B cells suggests that these findings may have therapeutic implications. Moreover, chlorambucil- and theophylline-induced apoptosis was consistently observed with the 15 samples of B-CLL cells tested.

Theophylline, a methylxanthine derivative, increases cAMP by blocking intracellular phosphodiesterase activity.²⁹ This was confirmed here in B-CLL cells, in which theophylline induced an accumulation of intracellular cAMP, a second signal required for apoptosis. The cAMP molecule is an ubiquitous second messenger that inhibits mature B- and T-lymphocyte proliferation³³ and induces the death of mature T lymphocytes³⁴ and thymocytes^{35,36} through internucleosomal DNA cleavage. Cyclic-AMP was recently shown to induce

apoptosis in resting human B lymphocytes³⁷ and germinal center B cells.³⁸ The important role of the cAMP pathway in regulating large numbers of genes through cAMP response elements, the cAMP responsive element modulator, or cAMP responsive element binding protein³⁹ may explain the effects we observed on bcl2, c-myc, and p53 expression during theophylline-induced apoptosis (see below).

Chlorambucil, which alkylates DNA, also strongly induced B-CLL apoptosis as previously reported.⁶ In contrast to theophylline, chlorambucil had little, if any, effect on intracellular cAMP levels, but elicits a sustained increase in intracellular calcium influx into B-CLL cells. This is the first demonstration of calcium influx involvement in chlorambucil signaling. The precise mechanism(s) by which chlorambucil promotes calcium influx is unclear and experiments are in progress to explain this phenomenon. Conflicting results have been reported on the link between an elevation of cytosolic calcium and apoptosis in normal and leukemic lymphoid cells.⁶ Studies on immature thymocytes have documented a critical role of a transient increase in cytosolic calcium in apoptosis signaling.³⁹ Robertson et al⁷ showed that incubation of B-CLL cells with fludarabine, a nucleoside analog commonly used to treat B-CLL, induced calcium influx associated with apoptosis and that treatment with a potent chelator of intracellular calcium resulted in total inhibition of DNA nucleosomal cleavage, in contrast to the chelation of the extracellular calcium. In the present study, we observed a moderate effect of intracellular calcium chelation on reversal of programmed cell death and only a partial effect of a calcium ionophore on its induction. These findings point to a role of other mechanisms in the observed apoptosis, such as the protein kinase pathways, which are involved in the rescue of germinal center B cells from apoptosis^{40,41} and in the apoptosis-induction of human B lymphocyte precursors by ionizing radiations.⁴² We observed a gradual increase in intracellular cAMP levels after chlorambucil treatment. As previously reported, calcium and cAMP have many interactions in several cell types. Cytosolic calcium, by binding calmodulin, induces adenylate cyclase activation which, in turn, catalyzes the formation of cAMP.^{29,43,44} Theophylline and chlorambucil thus appear to synergize through at least two distinct transduction pathways in B-CLL cell apoptosis. These results support those of a study showing chemosensitization to alkylating agents by pentoxifylline, another methylxanthine derivative, in CLL and acute myeloid leukemia.⁴⁵ A simple additive effect on B lymphocytic malignant cell apoptosis has also been described by Frankfurt et al⁴⁶ with a combination of chlorambucil and fludarabine, both of which induce calcium mobilization.

IL-4 acts as an antiproliferative cytokine in B-CLL⁴⁷ and B-acute lymphoblastic leukemia (B-ALL).⁴⁸ It also has an antiapoptotic effect in B-CLL,^{13,49} but can apparently induce apoptosis in B-ALL.⁵⁰ In our hands, IL-4 clearly inhibited theophylline-induced apoptosis in all 15 B-CLL samples tested and has a similar but less marked effect on chlorambucil-induced apoptosis. It is now established that IL-4, which has a specific receptor on human B lineage cells, even in the early steps of differentiation,⁵¹ stimulates tyrosine phosphorylation of various substrates.⁵² Our data suggest a link

between cAMP and tyrosine kinase pathways in the regulation of apoptosis. Furthermore, we confirm the role of IL-4 as an antiapoptotic molecule in B-CLL, and these results emphasize the physiological aspect of cAMP pathway in theophylline-induced programmed cell death.

Bcl-2, c-myc, and p53 are among the most important genes involved in induction or suppression of apoptosis.¹⁶ It has been reported that bcl-2 expression is constitutively higher in B-CLL cells than in normal B lymphocytes¹⁹ and has been shown to inhibit apoptosis in a large variety of cell types including B cells.⁴ Our data clearly indicate that both drugs downregulate bcl-2 gene transcription and protein expression in B-CLL cells. As previously reported,¹³ clear upregulation of bcl-2 expression and cell survival were observed after IL-4 treatment, before and after incubation with theophylline or chlorambucil. This is in keeping with early reports of the potent role of bcl-2 in reversing spontaneous⁵³ and chemotherapy-induced^{30,32} apoptosis. We have not found significant correlation between a spontaneous level of expression of bcl-2 before treatment and the susceptibility of B-CLL cells to apoptosis-inducing therapy contrary to a previous report in myeloid leukemic cells.²³

Theophylline- and chlorambucil-induced apoptosis also correlated with a significant increase in c-myc expression. The role of c-myc in inducing apoptosis²⁴ and the cooperative interaction with bcl-2 has previously been described in Rat-1 fibroblasts^{54,55} and hematopoietic pre-B cells.⁴ Harrington et al,⁵⁶ working on fibroblasts, suggested the involvement of c-myc in the induction of apoptosis and demonstrated that IL-4 can reduce the overexpression of c-myc and increase bcl-2 expression, thereby inhibiting apoptosis. As shown here, upregulation of c-myc proto-oncogene expression occurs concomitantly with downregulation of bcl-2 proto-oncogene expression. In contrast, theophylline and chlorambucil differ in their effect on p53. While theophylline downregulates wild-type p53 expression, chlorambucil elicits its overexpression. The p53 tumor-suppressor gene encodes a nuclear phosphoprotein that has transcriptional activation domains and specific DNA-binding capacity.⁵⁷ Wild-type p53 has been described as an inducer of apoptosis⁵⁸ and mutant p53 as a suppressor.⁵⁹ Kastan et al⁶⁰ showed that DNA-damaging agents induced an increase in wild-type p53 expression and cooperation between c-myc and p53 in apoptosis has been demonstrated by Hermeking et al.⁶¹ These results are in keeping with our analysis of apoptosis induced by chlorambucil alone and combined with theophylline. In contrast, the decrease in p53 expression observed during theophylline-induced apoptosis suggests that p53 does not play a role in this pathway of programmed cell death. This is supported by results from Clarke et al,⁶² who have demonstrated the role of p53 in radiation-induced thymocyte apoptosis, but not in glucocorticoid-induced apoptosis. In the same way, apoptosis induced by the retinoid N-(4-hydroxyphenyl) on the B cell lines K422 and DoHH2 and the myeloid cell lines HL60, and NB4 is not associated with changes in p53 protein levels.⁶³ According to Schwartz and Osborne⁶⁴ these data suggest that genes involved in cell death differ from one pathway to another. It is interesting to note that the increase in p53 expression after theophylline-

chlorambucil treatment might account for the synergy between the two drugs. Theophylline and chlorambucil modulate expression of genes closely connected in programmed cell death: induction of wild-type p53, enhancement of c-myc and suppression of bcl-2 are the consequences of theophylline-chlorambucil combination treatment and the conditions required to induce B-CLL apoptosis.

Methylxanthine derivatives and chlorambucil may thus have potential as a new therapeutic combination in B-CLL. The cAMP pathway appears to play an important role, along with the calcium pathway, and our results suggest a synergistic effect on oncogenes involved in B-CLL cell apoptosis. We recently tested this combination in a nonrandomized trial involving 12 patients with progressive or recurrent aggressive CLL after standard chemotherapy and observed rapid and marked responses in 11 cases using doses of an alkylating agent from threefold to 38-fold lower than in previous courses.⁶⁵ Nevertheless, the present work shows a new intracellular approach in understanding the mechanisms involved in B leukemia cell apoptosis and further experiments are in progress to test other B-cell malignancies.

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